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13. ABSTRACT *(Maximum 200 words)*

The goal of our research is to elucidate the role of a novel class II tumor suppressor, tropomyosin-1 (TM1) in breast cancer. Under the four technical objectives, we have initiated work on whether TM1 expression is altered during malignant transformation of mammary epithelium, and if those changes are causally linked to breast cancer. Expression of TM1 was assessed in normal and malignant breast epithelium. A novel TM1-specific antibody was developed, and with this reagent, by immunohisto-chemistry, we found that normal breast epithelium expressed TM1, while the cancerous tissue lacked TM1 expression (Objective 1). MCF-7 cells, which lost TM1 expression, were transduced to re-express TM1; restoration of TM1 expression results in altered morphology and in decreased growth rates. Both parental MCF-7 and the transduced clones remain sensitive to estrogen. Most significantly, expression of TM1 completely abolishes the growth of MCF-7 cells in soft agar. This demonstrates that TM1 abolishes the malignant phenotype MCF-7 cells and reverts them to normal phenotype. Therefore, loss of TM1 expression is a critical change during mammary carcinogenesis (Objective 2). Antisense experiments (Objective 3) and construction of chimeric TMs (Objective 4) are now in progress.

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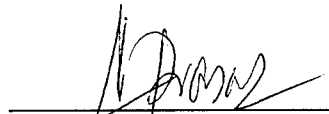

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Introduction

Title: Tropomyosin-1, A Putative Tumor Suppressor and a Biomarker of Breast Cancer.
Grant #: DAMD17-98-18162.

My research project investigates the role of a cytoskeletal protein, tropomyosin-1 (TM1) in breast cancer. TM1 is a member of tropomyosin (TM) family of microfilament associated proteins that bind to actin. Previous research from this and other laboratories has shown that TMs are downregulated in transformed murine fibroblasts. My work demonstrated severe and consistent derangements in TM expression in many breast cancer cell lines. Most significantly, TM1 expression was completely abolished in human breast carcinoma cell lines. Furthermore, TM1 was found to suppress the malignant phenotype of ras-, and src-transformed fibroblasts. Our work demonstrated that TM1 is a putative tumor suppressor. In this project, specifically I examine whether TM1 is suppressed in the primary tissue specimens obtained from breast carcinoma patients and whether TM1 can suppress the neoplastic phenotype of breast carcinoma cell lines.

Annual Summary

Title: Tropomyosin-1, A Putative Tumor Suppressor and a Biomarker of Breast Cancer.
Grant #: DAMD17-98-18162.

1. TM1 expression in normal and malignant breast tissue: Multiple isoforms of TMs are expressed in epithelial cells. In addition to TM1, six other TMs are expressed in human mammary epithelial cells, and the available antibodies and the cDNA probes do not permit accurate measurement of TM1 expression. This is due to extensive sequence homology among TM isoforms; and that TM1 expressed in epithelial cells, fibroblasts present (in stroma) and smooth muscle tissue is identical. To that end, we developed a TM1-specific anti-peptide antibody and demonstrated its specificity and utility in assessing TM1 expression in both human mammary cell lines and tissue specimens. This antibody was used for TM1 expression. Analysis of six specimens obtained from normal breast tissue showed that TM1 is expressed in the epithelial cells of all the specimens. But, in 6 specimens of invasive adenocarcinoma, the cancerous epithelium lacked the expression of TM1. Now, we are in the process of including more specimens.

However, when a different batch of antibody was employed, we discovered that in addition to recognizing TM1, the antibody cross-reacted with an unrelated 55-60 kDa protein. To obviate this unexpected problem, we used immunoaffinity chromatography to purify the TM1 antibody. In brief, the antigenic peptide was conjugated to CNBr activated sepharose, and the anti-TM1antiserum was passed through to bind the specific antibodies. After washing off non-specifically adhered proteins, anti-TM1 antibody was eluted by a combination of high and low pH washes. The affinity purified antibody was found to be highly specific, with no cross-reactivity to any other proteins and detected TM1 at dilutions >1:5,000, as tested in immunoblotting. At present we are using this immunoaffinity pure antibody to assess TM1 expression in tumor specimens. It is anticipated that we will complete this screening within 6-8 months. Then, we will assess TM1 expression in pre-malignant stages, such as DCIS, to identify earlier stages at which TM1 expression is lost or diminished. These studies will help in devising appropriate treatment strategies for breast cancer patients.

2. Effects of expression in human breast carcinoma cell lines: Our previous research demonstrated that TM1 is a class II tumor suppressor and its expression is consistently abolished in all the human breast carcinoma cell lines. To test whether TM1 suppresses the malignant phenotype of breast cancer cells, we chose to restore the expression of TM1 in MCF-7 cells. MCF-7 cells were transduced with a retroviral vector designed to express TM1 (MCF-7/T1). The transduced cells were selected in presence of G418 and clonal cell lines were established. Along with this, vector control cell lines of MCF-7 cells (MCF-7/v) were also established. TM1 expression was quantified in all the cells. MCF-7/T1 cells expressed TM1 in significant amounts, while the parental and vector control cells lacked TM1. We also detected TM1 in the cytoskeletal compartments of MCF-7/TM1

cells. We are in the process of measuring steady-state amounts of TM1 in the cytoskeleton.

Morphologically, TM1 expression in MCF-7 cells induced a more 'branched' cell growth in two-dimensional cell cultures. To test whether TM1 induces a more differentiated organoid structures, three-dimensional cultures in matrigel are planned. Restoration of TM1 expression in MCF-7 cells, decreases the growth rates of MCF-7 cells.

A more definitive suppressive effect of TM1 on the neoplastic phenotype pertained to the inability of MCF-7/T1 cells to grow under anchorage-independent conditions, which is an important concomitant of the transformed phenotype. The parental MCF-7 and the vector control MCF-7/V cells grew and formed colonies with equal efficiency in soft agar assays. But, MCF-7/T cell lines were unable to grow and form colonies in soft agar. Thus, re-expression of TM1 in MCF-7 cells suppresses the transformed phenotype.

Experiments are in progress to further characterize the revertant phenotype of MCF-7/T1 cells. The revertant MCF-7/T1 and MCF-7 cells are responsive to estrogen in terms of their rate of proliferation. Tamoxifen decreases the growth rates of all the cell lines, and the addition of estrogen overcomes the inhibitory effects of tamoxifen. To biochemically characterize the estrogen responsiveness of the cell lines, we are testing the levels of hormone receptors of estrogen and progesterone, and the expression of estrogen-inducible protein, pS2.

In a separate study, we previously discovered an epithelial marker protein, HME1 (14-3-3σ), which is down regulated in breast carcinoma cell lines, including in MCF-7. To assess the degree of differentiation, expression of HME1 is quantified in the TM1-revertants of MCF-7 cells by Northern blotting. MCF-7 cells expressed significantly lower amount of HME1 RNA than normal MCF-10A cells. In the MCF-7/T1 cells, expression of HME1 is enhanced nearly to the levels found in MCF10A cells, indicating that TM1 induced reversion involves upregulation of differentiation-associated proteins.

We have concentrated on this objective to determine whether TM1 is causally linked to mammary carcinogenesis. Accumulating data from objectives 1 and 2 strongly suggest that loss of TM1 is a pivotal event in the neoplastic transformation of breast epithelium. Thus, TM1 could serve as a biomarker in the clinical management of breast cancer, and it appears to be a suppressor of the malignant phenotype of breast cancer cells.

3. Induction of transformed phenotype by suppression of TM1 expression: Experiments to specifically suppress TM1 in normal MCF10A are now initiated. TM1 was subcloned into pBNC retroviral vector in antisense orientation and the infectious retroviral supernatants are now being generated. For this purpose, amphotropic packaging cell line, PA317, was employed and was transfected with the recombinant 'TM1-as-pBNC' vector which expresses full length TM1 mRNA in antisense orientation. Transfected cells were selected in presence of G418 and the colonies were isolated by cloning cylinders. Infectious supernatants are now being generated. These supernatants will be used for infection of the target MCF10A cells.

4. Structure-function relationship of TM1-mediated tumor suppressive effects: Work on creating chimeras of TM1 (a tumor suppressor) and TM2 (not a tumor suppressor) has been initiated. We have completed the site directed mutagenesis to introduce a silent mutation to create a HindIII restriction site. This was accomplished by PCR and the resultant variants of TM1 and TM2, designated as 'TM1-h' and 'TM2-h' respectively, containing the HindIII site were sequenced. Switching of the carboxy (at AvaI) site and the central exons (HindIII-AvaI) is now in progress. This is in line with the objectives listed in the statement of work, Task 1. We expect to make rapid progress in terms of the construction of chimeras and their expression in the next few months.

Appendix

Title: Tropomyosin-1, A Putative Tumor Suppressor and a Biomarker of Breast Cancer.
Grant #: DAMD17-98-18162.

Key Research Accomplishments:

- Specificity of anti-TM1 antibody was significantly improved.
- Immunoscreening of breast tumors for TM1 expression is in progress.
- TM1 expression is absent in invasive carcinomas.
- MCF-7 cells were transduced to express TM1.
- Restoration of TM1 expression reverts MCF-7 cells to normal phenotype.

Reportable Outcomes:

1. One manuscript entitled, "Suppression of neoplastic transformation and regulation of cytoskeleton by tropomyosins. *Somatic Cell and Molecular Genetics.* Authors: Vanya Shah, Richard Braverman, and **G. L. Prasad**. (in press) (1999)."
2. Two abstracts were presented:
   2.1. **Prasad, G. L.** and Shah, V Suppression of the transformed phenotype by Tropomyosin-1, a class II tumor suppressor. (1999) 99th Annual meeting of the American Association for Cancer Research. Abstract # 4612.
   2.2. **Prasad, G. L.,** Hyland, L. J., Caya, J. G., and Raj, M. H. G. Tropomyosin-1, a class II tumor suppressor in breast cancer. (1998) Breast Cancer Res. Treat. (Abstract # 577) 50: 335.
3. We developed MCF-7 cell lines that express TM1.
4. Many grant applications were submitted based on the support of this award.
   4.1. *US Army Breast Cancer Research Program:* For $316, 050 (July 1999-June 2002). P. I. G. L. Prasad. Title: Tropomyosin-1: a novel class II tumor suppressor and a biomarker of breast cancer.
   4.2. *Pennsylvania Department of Health*: For $25,000 (January 1999 to December 31 1999) P. I.: G. L. Prasad. Title: Tropomyosin-1: A Tumor Suppressor and a Novel Biomarker of Breast Cancer.
   4.3. *American Heart Association:* Regulation of Cytoskeleton by Tropomyosin-1.
   4.4. *US Army Breast Cancer Research Program:* HME1 (14-3-3σ), a Regulator of Growth and Differentiation of Mammary Gland.
   4.5. *American Cancer Society*: Molecular Mechanisms of Tumor Suppression by Tropomyosin-1.
5. Copies of the abstracts and manuscripts are enclosed.

Suppression of Neoplastic Transformation and Regulation of cytoskeleton by Tropomyosins.

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Running Title: Tropomyosins and cell transformation

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Abstract:

Down regulation of Tropomyosins (TMs) is a consistent biochemical change observed in many transformed cells. Our previous work has demonstrated that Tropomyosin-1 is an anti oncogene and it is a class II tumor suppressor. Using ras-transformed murine fibroblasts (DT cells), we have examined the effects of co-expression of two isoforms of TM on cell morphology, cytoskeleton and tumorigenecity. Enhanced expression of TM1, a suppressor of transformation, along with TM2 which is not a tumor suppressor results in the formation of well-organized microfilaments, a morphology that resembles normal fibroblasts, and suppression of tumorigenecity. Tumor formation *in vivo* was compatible with the persistence of high-level of TM2, but not TM1. Homodimers of TM1 and TM2 were observed in these cells. Thus, restoration of expression of TM1 and TM2 proteins in *ras*-transformed cells suppresses the transformed phenotype with dramatic re-organization of microfilaments. These data show that TM2 cooperates with TM1 in the reogranization of microfilaments, while TM1 is a suppressor of the transformed phenotype.

Introduction:

Tropomyosins are a family of cytoskeletal proteins which bind to and stabilize actin in the microfilaments (1,2). The expression of TMs is regulated in a highly tissue-specific manner by alternative splicing mechanisms. Although the functions of these proteins are well documented in muscle contraction, their role in non-muscle cells is less clearly understood. Non-muscle cells express multiple isoforms of TMs, which are categorized as high and low M_r species, compared to one or two isoforms (high M_r TMs) found in muscle cells. TMs have been suggested to participate in many diverse physiological processes, in addition to the stabilization of microfilaments against the actions of gel-severing proteins (3). It remains to be determined whether all non-muscle TM isoforms perform distinct physiological functions, or they are functionally redundant; however, recent work suggests that individual TMs may perform defined functions (1,2).

The expression of non-muscle isoforms is down-regulated in many transformed cells (4-6). In fact, the loss of high M_r TMs is shown to be a common feature of cells transformed by multiple transforming modalities (5,6,7), including that of some human malignant cell lines (8,9). For example, in *ras*-transformed NIH3T3 (DT) cells, TM1 expression is down regulated to 50% of levels found in normal NIH3T3 cells (5,10), while breast carcinoma cell lines completely lack the expression of TM1 (7). This led to the hypothesis that the suppression of TMs by oncogenic modalities results in the assembly of defective microfilaments (6,11), which in turn would contribute to the malignant phenotype. In support of this hypothesis, data from this laboratory demonstrated that restoration of the expression of tropomyosin-1 (TM1) results in suppression of the transformed phenotype induced by *ras* and *src* oncogenes (10-12).

Furthermore, tumor suppression by TM1 is isoform-specific (11).

Although restoration of TM1 expression was sufficient to suppress the transformed phenotype of *ras*-transformed fibroblasts (10), these cells still lacked another prominent TM isoform, TM2, which did not function as a tumor suppressor of DT cells. The revertant cells, which overexpress TM1 exhibited flat morphology and manifested restored cytoskeletal architecture. Yet, the microfilaments were not completely restored to the extent found in normal NIH3T3 cells. Since TMs are known to interact as dimers, we investigated the possibility that TM2 may also contribute in the assembly of microfilament bundles. In this communication, we describe the effects of re-expression of TM1 and TM2 in DT cells. We examined the morphology, tumorigenesis and TM interactions in this study. Our data indicate that TM2 cooperates with TM1 in microfilament organization.

Materials and Methods:

Normal NIH3T3, transformed DT cells, DT/TM1 and DT/TM2 cells have been previously described (10). Expression of TM1 (13) in DT cells results in the suppression of transformed phenotype (10). These cells were transfected with a TM2 expression plasmid (TM2-pEE6) in which TM2 expression is driven by CMV promoter (11). The cells were selected with mycophenolic acid (Sigma Chemical Co., St. Louis, MO) essentially as described previously. The resulting cells were maintained in G418 (Life Technologies, Gaithersburg, MD)(200μg/ml) and mycophenolic acid (20μg/ml), and cloned (10, 11).

The procedures for Northern blotting, Western blotting and two-dimensional gel electrophoresis were described elsewhere (12). Soft agar and tumorigenesis in athymic nude mice were performed using previously published protocols (11). Cross linking of TMs (14,15) was performed with some modifications. The principle of cross linking was previously described in detail (16). Cell lysates were treated with the sulfhydryl cross-linking agent 5,5'-ditho-bis-(2-nitrobenzoic acid) (NbS_2) (Sigma) at 20^0C for one hour and subjected to SDS-PAGE under reducing and non-reducing conditions, by the addition or omission of 2-mercapto-ethanol (Et-

SH), respectively. The cross linking reaction is complete under these conditions as prolonged incubation with NbS_2 does not result increase the yield of homodimers (data not shown). Treatment with the reducing agent cleaves the cross bridges formed by the cross-linker, while its absence in the gel sample buffer permits the detection of homo dimers of TMs.

The cytoskeletons of cultured cells were observed using confocal microscopy (12). Cells were cultured in chamber slides (Labtek, Naperville, IL), rinsed with phosphate buffered saline (PBS), fixed in 3.7% paraformaldehyde in PBS and extracted with 0.5% triton x-100 for 30 min. Then, they were reacted with an antibody that recognizes all TM isoforms, followed by incubation with FITC conjugated phalloidin (Molecular Probes, Eugene, OR). To observe actin filaments, phalloidin coupled to Texas Red (Molecular Probes) was added to the samples and mounted with Slow fade (Molecular Probes) according to the manufacturer. Images were viewed with a laser scanning confocal microscope (Carl-Zeiss) and the composite three-dimensional images were projected.

Results and Discussion:

Previous studies (10) demonstrated that enhanced expression TM1 protein by retroviral mediated gene transfer reverts DT cells to normal growth phenotype. These cells still lacked TM2. A stable revertant cell line was used for restoration of TM2 expression. TM2 cDNA, cloned in TM2-pEE6 vector was transfected into DT/TM1 cells. The stable cell lines were isolated by selection with mycophenolic acid and G418. The expression of TM1 and TM2 was detected in all the cell lines. TM expression was monitored by Northern blotting, and two-dimensional gel electrophoresis as well as by immunoprecipitation and immunoblotting (data not shown). TMs are identified based on their mobility on two-dimensional gels and immunoreactivity, as determined in our earlier studies (10, 11). It should be noted that both endogenous TM1 and the transduced TM1 proteins have identical electrophoretic mobilities due to sequence homology.

In the DT/TM1-TM2 cells, TM1 is expressed from two different mRNAs: the endogenous 1.1 kb and the transduced 2.0 kb RNAs code for TM1 (10). In DT cells, the

expression of endogenous 2.0 kb TM2 mRNA is abolished due to the oncogenic action of *ras*. Therefore, the 2.0kb mRNA detected in these cells with DT back ground represents transduced TM1. Transfection of DT/TM1 cells with TM2-pEE6 plasmid results in the expression of a 1.3 kb mRNA which encodes TM2 protein. An oligonucleotide probe, complementary to the first 30 bases of TM1 (identical in TM2 also), detects all three mRNAs (Fig. 1A). The sizes and the levels of expression of TM mRNAs are as expected and are consistent with our previous data (11). Depending upon the cell line the degree of expression of the endogenous TM1 mRNA was variable. In Fig. 1B, expression of TM1 and TM2 is shown in the heat stable preparations of lysates from metabolically labeled DT/TMl-TM2 cells. Expression of TM1 and TM2 are evident in this representative two-dimensional autoradiogram. In the control cells which were generated by mock-transfection, only TM1 is found. Quantitative data from a number of individual cell lines is presented in Fig. 1C. Among the individual cell lines TM2 expression was variable, resulting in different ratios of TM1 to TM2 proteins. We utilized DT/TM1-TM2-1 and DT/TM1-TM2-2 cell lines for further analysis.

Morphologically, DT/TM1-TM2 cells are flat, with well-spread appearance and well-organized microfilaments. These cells resemble NIH3T3 cells in appearance. This is interesting, because, DT/TM1 cells are reverted and are flat in appearance with a limited degree of organized microfilaments. The DT/TM2 cells, on the other hand, remain transformed. Although, expression of TM2 by itself does not revert DT cells, its co-expression with TM1 improves the morphology and microfilament organization. This is evident from the microfilament organization of various cell lines, as shown in Figure 2. Normal NIH3T3 cells contain well defined microfilaments, and TMs colocalize along the microfilaments. In DT cells, which were stained for TMs alone (green), no defined organization of actin filaments is evident, and the TMs are dispersed in

cytoplasm. Restoration of TM1 results in improved morphology and cell spreading and a limited re-emergence of the microfilament structures, as evidenced by TM staining. On the other hand, expression TM2 cDNA in DT cells, does not lead to re-appearance of actin filaments, as observed by double fluorescence of phalloidin and TM staining. Transfection of DT/TM1 cells with TM2 to express both the TM isoforms results in the formation of prominent stress fibers. Thus, TM2 appears to cooperate with TM1 in remodeling the cytoarchitecture of transformed fibroblasts.

The effects of co-expression of TM2 on the revertant phenotype of DT/TM1 cells were not readily evident. Since TM1 expression inhibits the transformed phenotype, the cells that express TM1 and TM2 remain reverted. The DT/TM1-TM2 cells do not grow under anchorage independent conditions, as judged by soft agar (data not shown). Similarly, tumorigenesis experiments in athymic nude mice indicated that tumor growth was not compatible with the continued expression of TM1. The mice inoculated with DT/TM1 cells formed tumors with a delay, but eventually all the mice were sacrificed due to the tumor burden. When the tumors were analyzed for the expression of TM1 by Northern blotting, the transduced 2.0 kb TM1 mRNA was found to be completely lacking (Fig. 3, lanes 2 and 4). These data are in agreement with our previous results that show transduced TM1 expression is extinguished in the animals and thereby tumor growth is facilitated (10,11). However, low level of TM1 is still produced as evident from the persistent expression of the endogenous 1.1 kb mRNA.

TM2 was also extinguished in tumors derived from DT/TM1-TM2-2 cells (Fig.3, lane 4) and other DT/TM1-TM2 cell lines (data not shown). However, it was observed that tumors that arose after inoculation with DT/TM1-TM2-1 cells grew more aggressively relative to those from other cell lines (Fig. 3). Analysis of these tumors revealed that TM2 mRNA is expressed at high

levels, with no detectable transduced TM1; the endogenous TM1 mRNA was present, however. The TM2 mRNA expression in the tumors is greater than that in the cultured cells, indicating selective up-regulation of TM2 occurred in these tumors. We have also found that continued TM2 expression in many tumors arising from independent inoculations of DT/TM1-TM2-1 cells (data not shown). This suggests that the integration of TM2 cDNA occurred in a more permissive environment in the DT/TM1-TM2 cells to facilitate the in vivo expression. More significantly, tumor growth is clearly possible in presence of TM2, but not when TM1 is expressed.

In vivo TMs exist as dimers. Earlier in vitro experiments have suggested that heterodimeric interactions among TMs, eg., TM1:TM2, are thermodynamically preferred and may in fact be more effective in stabilizing microfilaments (14,15). Our previous work indicated that homodimers of TM1 are found in the revertant DT/TM1 cells (16). We hypothesized that these homodimers of TM1 are responsible in reverting DT cells and in the re-organization of the cytoskeleton (16). To test whether the presence of TM2 influences TM1 interactions under steady-state conditions, we have cross-linked the cell lysates with NbS_2 and performed immunoblotting to determine TM interactions. The identity of the dimeric species is established by excising them and re-electrophoresing under reducing conditions (16). In agreement with our previous data wherein we utilized immunoprecipitation of pulse-labeled proteins for crosslinking, we detect TM1 homodimers (Fig. 4, right panel). Homodimers of TM1 and TM2 were found in NIH3T3 and DT/TM1-TM2 cells. It is likely that the homodimers of TM1 continue to be critical in maintaining the stable, reverted phenotype. In addition to TM1 homodimers, we also detected TM2 homodimers in DT/TM1-TM2 cells where TM2 is also available. In DT and DT/ TM1 cells, only homodimers of TM1 are found. These data are consistent with previous observations from our laboratory (16) as those of other investigators

by excising them and re-electrophoresing under reducing conditions (16). In agreement with our previous data wherein we utilized immunoprecipitation of pulse-labeled proteins for crosslinking, we detect TM1 homodimers (Fig. 4, right panel). Homodimers of TM1 and TM2 were found in NIH3T3 and DT/TM1-TM2 cells. It is likely that the homodimers of TM1 continue to be critical in maintaining the stable, reverted phenotype. In addition to TM1 homodimers, we also detected TM2 homodimers in DT/TM1-TM2 cells where TM2 is also available. In DT and DT/ TM1 cells, only homodimers of TM1 are found. These data are consistent with previous observations from our laboratory (16) as those of other investigators (17). It is generally considered that TM1 and TM2 heterodimers exist when both TM1 and TM2 are available. However, the present data do not distinguish between uncross linked monomers, and TM1:TM2 heterodimers Formation of TM1:TM2 heterodimers is not demonstrable in DT/TM1-TM2 cells from the data of Figure 3. Ongoing experiments will conclusively establish this. Nevertheless, the homodimers of TM2 may have a role in improving the organization of TM1-induced microfilamental architecture. In addition, the heterodimers of TM1 and TM2 also could be critical to the assembly of well-organized microfilaments and the stability of the cytoskeleton.

It should be noted that DT cells are highly tumorigenic in nude mice and aggressively grow as colonies under anchorage independent conditions. We reported that TM2 is unable to suppress the transformed phenotype of DT cells, and our present data also strongly support that conclusion. At variance with our data it was reported that TM2 could act as a suppressor of the ras transformation (18). These authors attributed the differences in the tumorigenic potential of the two parent cell lines as the reason for this apparent discrepancy. It is possible that TM2 in some way be co operating with the endogenous TM1 to suppress the weakly transforming cells

used in that study. Our data together with other published work using *v-ki-ras* transformed NRK cells (19) clearly demonstrate that TM2 does not function as a suppressor of the transformed phenotype. It was also shown that re-expression of TM2 results in cell-spreading in NRK cells (20). TM1, on the other hand, is suppressor of transformation and re-organizes the cytoskeleton independent of other TMs.

A number of other findings demonstrate the critical role of TMs in cell physiology. For example, in yeast, loss of a gene that encodes a major TM disrupts actin filaments, and results in viable, but unhealthy cells (21). Further loss of a second, but minor form of TM combined with the loss of the major TM isoform is lethal (22). Recent data also indicate that tropomyosin 3 plays a role in intracellular transport (23). Also TM is implicated in the localization of specific mRNAs during embryogenesis (24).

For complete reversion of the transformed phenotype, we hypothesize that TM1 modulates the intracellular signaling of the transforming oncogene and re-organize the cytoskeleton. There is evidence that signal transduction pathways play a role in regulating the assembly of microfilaments and the expression of TMs (25). In addition, the pathways regulated by Rho GTPases (26), most notably through Rho kinase (27) and or contraction driven by myosin light chain phosphorylation (28) are likely to play a critical role in microfilament organization. These pathways may be critical in TM1-induced cytoskeleton and the suppression of the transformed phenotype.

Another important aspect that emerges from this study is that the ratios of TM1 and TM2, are critical for formation of microfilaments. TM1 alone dramatically suppresses the transformed phenotype and restores microfilament structures partially, while TM2 expression in itself is inadequate to revert the cell phenotype and improve cytoskeleton. Continued expression of TM1

and TM2 appears is required for well-developed actin filaments.

Acknowledgements: This work is supported by grants from American Heart Association (Pennsylvania Affiliate), WW Smith Charitable Trust, Philadelphia and American Cancer Society (IRG #204) to GLP. GLP is also a recipient of a career development award (DAMD17-98-1-8162) from the US Army Breast Cancer Research Program.

The Abbreviations used are: kb, kilobases**;** TM, tropomyosin; TM1, tropomyosin isoform 1; TM2, tropomyosin isoform 2.

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Figure Legends

Figure 1: Expression of TM1 and TM2 in DT cells: **A**. Northern blotting of total RNA (20μg) isolated from DT (lane 1), DT/TM1 (lane 2) and DT/ TM1-TM2 cells (lane 3). The blots were probed with an oligonucleotide that is complementary to the first 30 nucleotides of TM1. This probe detects endogenous TM1 (1.1 kb), transduced TM1 (2. 0 kb) and TM2 (1.3 kb) mRNAs. **B**. Two-dimensional gel analysis of heat stable protein preparations of DT/TM1 and DT/TM1-TM2 cells. The positions of TM1 and TM2 are identified. Cells were labeled with [^{35}S]-methionine and the heat stable preparations were prepared and analyzed by two dimensional gels (5,7). **C**. A number of DT/TM1-TM2 cells were generated and expression of TM1 and TM2 was quantified on AMBIS radioanalytical imaging system (10-12) and the ratio of TM2/TM1 was presented.

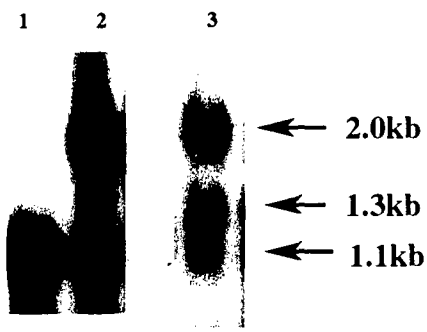
Figure 2: Microfilament organization: To visualize actin filaments, Texas Red conjugated phalloidin was used. TMs were localized by double immunostaining with FITC-conjugated second antibody. Co-localization of actin and TMs is indicated by a yellow color. The cell lines were grown on chamber slides and prepared for confocal microscopy as described in the Experimental Procedures.

Figure 3: Expression of TM2 in tumors: Athymic nude mice were injected with DT/TM1-TM2 cells, and the tumors were resected and analyzed for TM expression by Northern blotting with an oligodeoxynucleotide, as described in the experimental procedures. Twenty μg of total RNA extracted from the corresponding tissue culture cells is also included. Lanes 1, DT/TM1-TM2-1

cells; 2, DT/TM1-TM2-1 tumor; DT/TM1-TM2-2 cells; and, DT/TM1-TM2-2 tumors. The TM RNAs and their sizes are given in Figure 1.

Figure 4: Dimerization of TMs: Cell extracts (70μg) were prepared from the indicated cell lines and cross linked with NbS_2. Cross linked samples were run on SDS-PAGE either in the absence (right panel) or presence (left panel) of 2-mercaptoethanol (Et-SH), as indicated. Western blotting was performed using TM antibody. The position of TM monomers and dimers is indicated. Previously, we have analyzed the dimers and identified the individual TM species (16).

A.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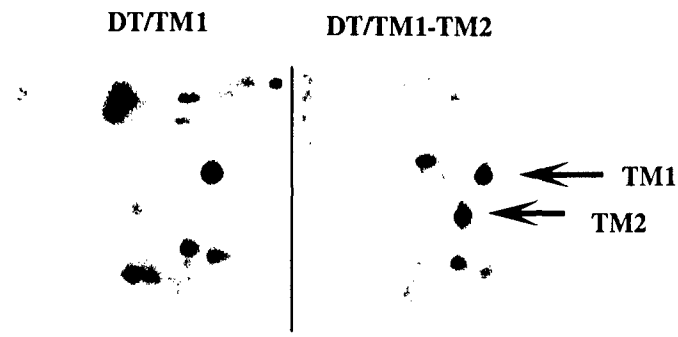
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TM1
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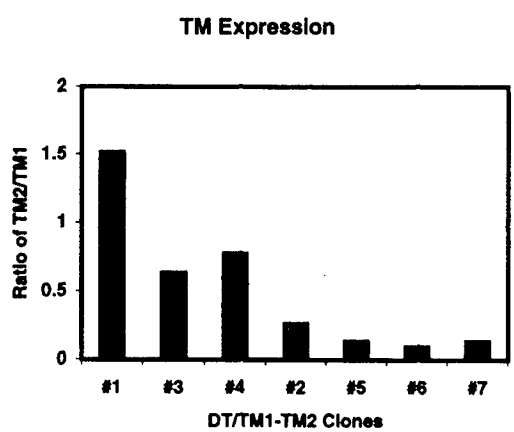
C.


TM Expression
Ratio of TM2/TM1
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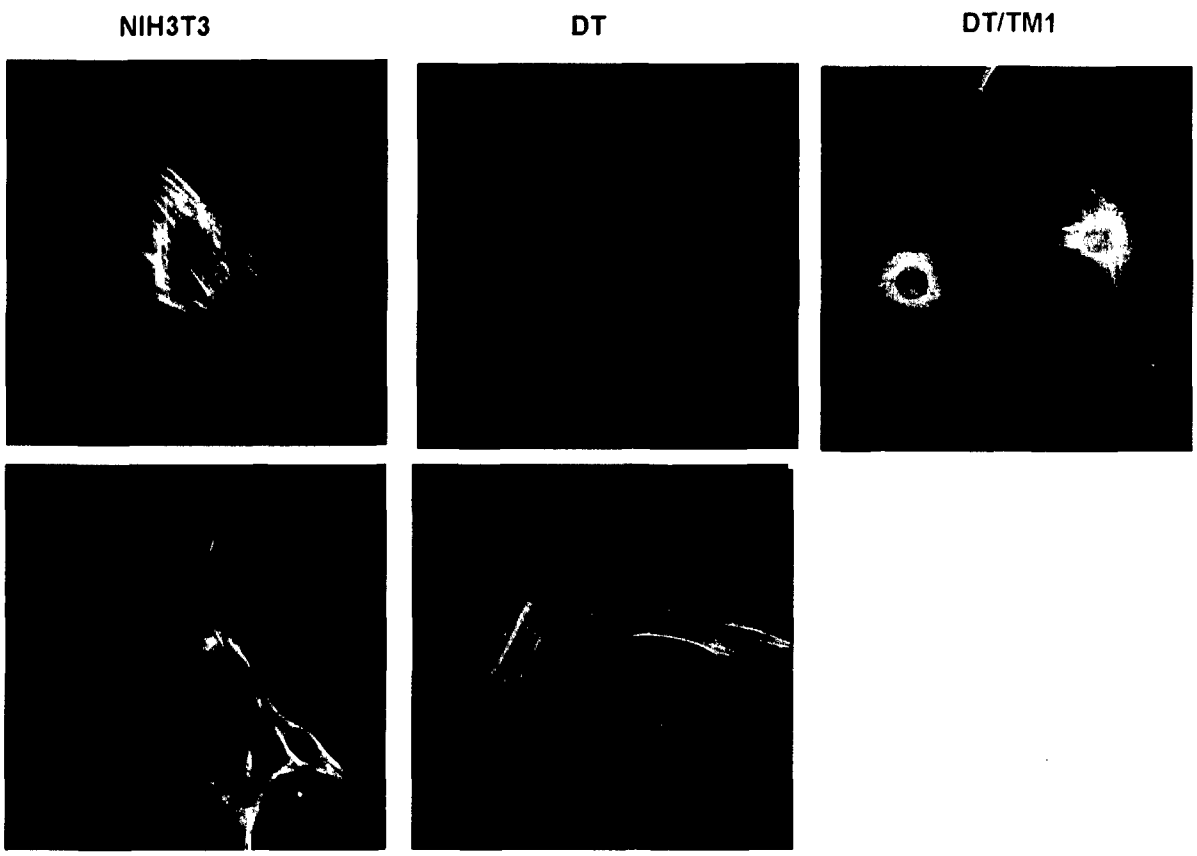
DT/TM1-TM2 Clones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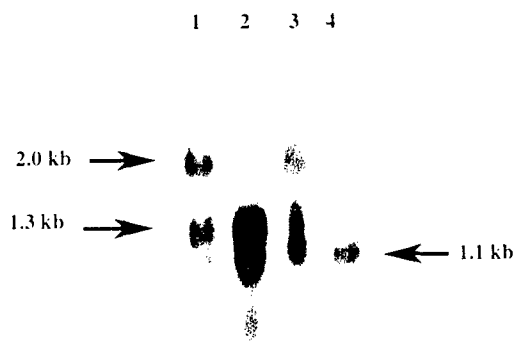
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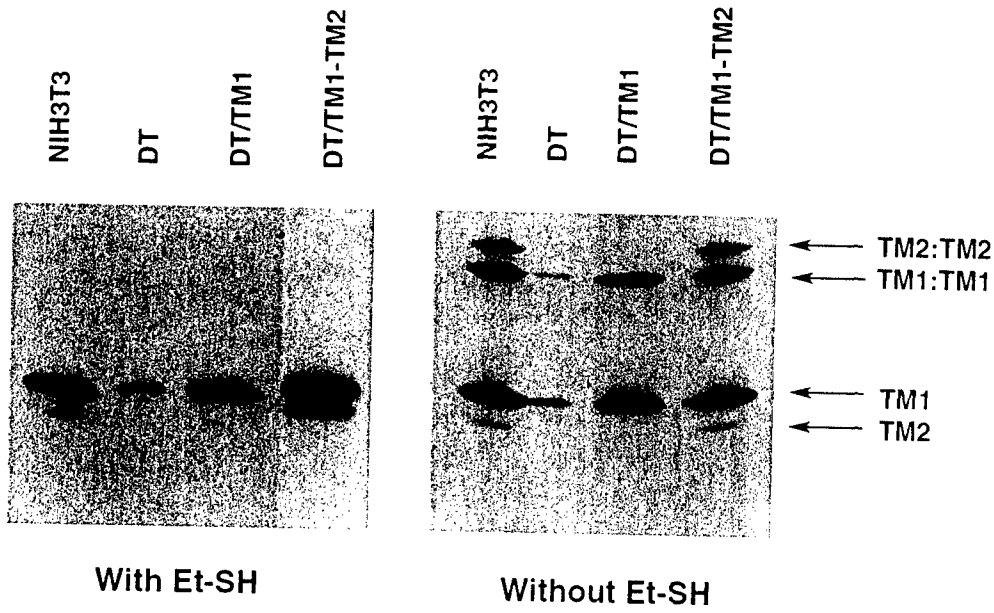
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TM1
TM2
With Et-SH
Without Et-SH

Prasad GL, Hyland LJ, Caya JG, Raj MHG.
Philadelphia PA; New Orleans LA.

Tropomyosin-1, a class II tumor suppressor in breast cancer.

Abstract # 577.

Previously, we identified that loss of tropomyosin-1 (TM1) expression is one of the pivotal biochemical events in the neoplastic transformation of cells. A decrease or loss of TM1 expression results in the formation of aberrant microfilaments, which may contribute to the acquisition of metastatic phenotype. Restoration of TM1 expression alone is adequate to suppress the transformed phenotype of cells transformed by functionally diverse oncogenes. Physiological levels of TM1 appear to be required for normal growth and differentiation. During the multi-step carcinogenesis, the expression of class II suppressors (*viz.*, TM1), which are targets of class I tumor suppressor proteins, is altered. Thus, modulation of class II suppressors may facilitate the neoplastic transformation.

We have demonstrated that TM1 expression is completely abolished in many transformed human breast carcinoma cell lines. These data indicate that TM1 suppression may be a common feature of neoplastic transformation of mammary epithelium. In this study, we have evaluated whether TM1 expression serves as a useful biomarker of breast cancer. To accomplish this, a novel TM-1 specific antibody is generated and TM1 expression in breast tumor specimens is immunohistochemically assessed. Normal mammary epithelium expresses abundant quantities of TM1 while the malignant tissue lacks any detectable TM1. Further experiments are in progress to determine the stage at which loss of TM1 occurs during course of neoplastic transformation of the mammary epithelium.

To investigate whether the loss of TM1 is causally linked to the mammary carcinogenesis, we have restored expression of TM1 in MCF-7 cells, which lack TM1. Experiments are in progress to determine the effect of TM1 re-expression on the morphology, cytoskeletal organization and the transformed phenotype of the cells. We expect to demonstrate that TM1 functions as a suppressor of the transformed phenotype of human breast cancer cells.

Introduction

Tropomyosins (TMs)

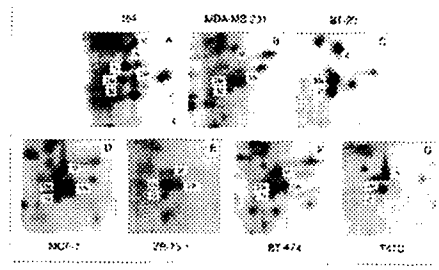
- Family of cytoskeletal proteins associated with actin microfilaments.
- Acidic, heat-stable proteins with extensive α-helical structure.
- Broadly classified as:
  a) high M_r isoforms (284 aa; 41-35 kDa) found in muscle and non-muscle cells.
  b) low M_r isoforms (248 aa; 30-32 kDa) found in non-muscle cells.
- Function:
  a) In skeletal muscle: part of Ca++-dependent acto-myosin contractile mechanism.
  b) In non-muscle cells: May stabilize microfilaments against f-actin severing factors.
- Expression of high M_r TMs is down regulated in transformed cells.
- We demonstrated that a high M_r isoform, *TM1, is a suppressor of malignant transformation* induced by oncogenes. It is a class II tumor suppressor.

In this study we examined the role of TM1 in Breast Cancer.

1. Is TM1 expression altered during mammary carcinogenesis?
2. Can TM1 suppress the malignant phenotype of breast carcinoma cell lines?

Fig. 1. Expression of TMs in Breast carcinoma cell lines.

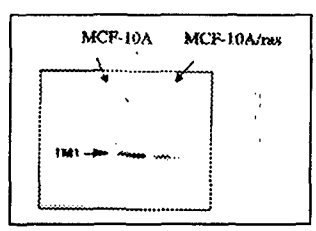
Heat-stable cell lysates from metabolically labeled cell lines were analyzed by 2-dimensional gel electrophoresis.



- Expression of one or more high M_r TMs is abolished in the malignant cell lines.
- TM1 is consistently absent in all the transformed cell lines.

Fig. 2. TM1 expression in mammary epithelial cells.

Western blotting of control and *ras*-transformed MCF10A cells. TM expression was analyzed by immunoblotting.



- TM1 expression is down regulated upon transformation by *ras*.
- This is consistent with the data of Fig.1 and our previous work demonstrating that transformed cells express reduced TM1.

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Expression of TMs in Breast ›ma cell lines.

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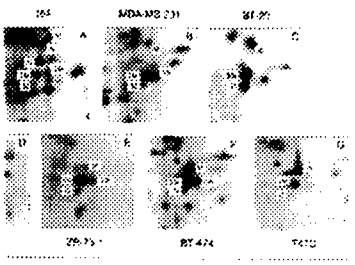

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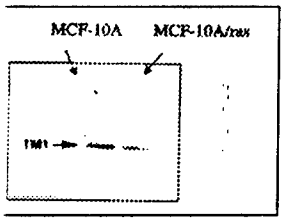

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Hypothesis

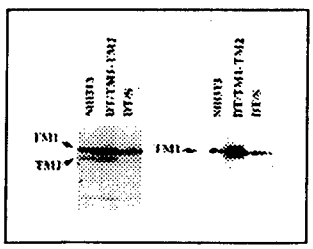
- The alterations in TM1 expression may be required for the initiation and/or progression of breast cancer.
- Multiple oncogenic modalities suppress TM1 expression. This results in the formation of defective microfilaments, which in turn contribute to the transformed phenotype.
- Thus, loss of TM1 expression is a common, yet critical event during cellular transformation.

Is TM1 expression abolished in the primary tumors?

To facilitate the analysis of TM1 expression in the primary tissue specimens obtained from breast cancer patients, we developed a novel TM1-specific antibody.

Fig. 3. Specificity of TM1-antibody.

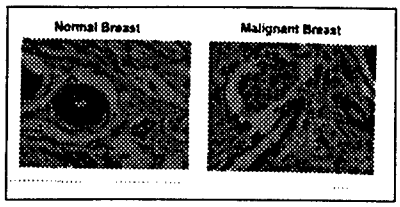
Western blotting of indicated cell lysates with TM antiserum that recognizes multiple TM isoforms (left panel), and the novel TM1-specific antibody (right panel). The cell lines have defined TM isoform expression.



- The antibody recognizes TM1 specifically, and does not cross-react with other TMs.

Fig. 4. TM1 expression in the tissue specimens.

TM1 expression was assessed in normal and in invasive ductal adenocarcinoma of the breast by immunohistochemistry, using a TM1-specific antibody.

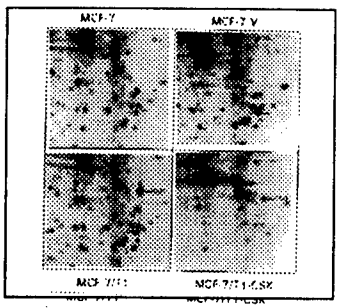


- TM1 is expressed in normal epithelium, but essentially absent in the carcinoma.

Can TM1 suppress the transformed phenotype of breast carcinoma cells?

Fig. 5. TM expression in MCF-7 cells.

MCF-7 cells were transduced to overexpress TM1.



- TM1 expression was restored in MCF7 cells.

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The alterations in TM1 expression may be required for the initiation and/or progression of breast cancer.

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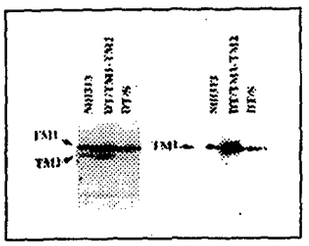

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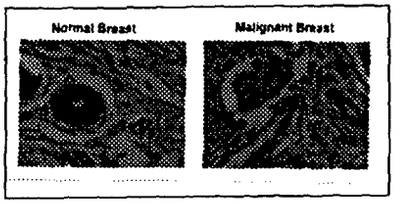

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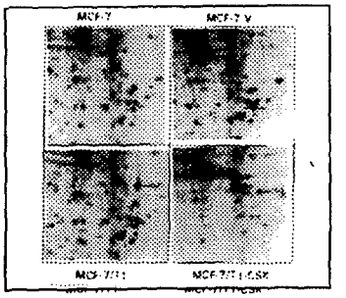
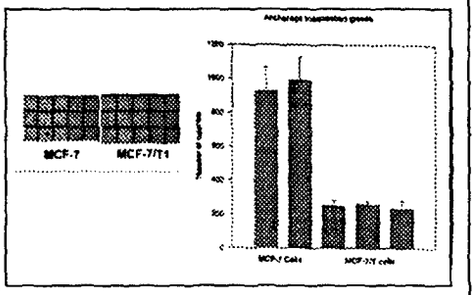


- TM1 expression was restored in MCF7 cells.

Fig. 6. Effect of TM1 expression on the transformed phenotype of MCF-7 cells.

Cells were cultured under anchorage independent conditions. Colonies greater than ≥ 50μm are scored.



- Restoration of TM1 expression cells significantly suppresses the transformed phenotype of MCF-7 cells.

Conclusions

1. TM1 expression is suppressed in breast cancer cell lines.
2. The initial data indicate that TM1 expression is abolished in the malignant breast tumor specimens obtained from patients. Loss of TM1 expression may be a stage-specific event during mammary carcinogenesis.
3. Detailed studies could show that TM1 is a novel biomarker of breast cancer.
4. The data suggest TM1 reverts the transformed phenotype of MCF-7 cells.

Acknowledgements:

This work is supported by grants from National Race for the Cure, American Cancer Society, W. W. Smith Charitable Trust, Philadelphia, and American Heart Association. GLP is also a recipient of a Career Development Award (DAMD17-98-1-8162) from US Army Breast Cancer Research Program.

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Suppression of the transformed phenotype by Tropomyosin-1, a class II tumor suppressor. Prasad, G. L. and Shah, V. *Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA19140*

Suppression of high M_r tropomyosins (TMs) is a common feature of the transformed cells. Previously, we demonstrated that TM1, one of the isoforms of TMs, is an anti-oncogene that belongs to the category of class II tumor suppressors. Understanding the mechanism of tumor suppression by TM1 has important implications. This knowledge is essential to develop novel therapeutic strategies to cancer, and to learn how the interactions of the cytoskeleton and signal pathways control growth and differentiation. An important aspect of TM1-mediated suppression of the transformation would involve TM interactions with other cytoskeletal proteins, and homodimeric or heterodimeric associations of TMs. Furthermore, modulation of the intracellular signal transduction pathways will be a major mechanism by which TM1 suppresses the transformed phenotype. We have utilized ras-transformed fibroblasts (DT) as the model system to test the above hypotheses. To facilitate these studies, we created cell lines of DT with defined TM expression profiles and stable phenotypes. Co-expression of TM1 and TM2—a closely related TM isoform, but not a tumor suppressor—in DT cells results in improved cell morphology and cytoskeleton with the cells reverting to normal phenotype. By generating chimeras of TM1 and TM2, we expect to identify and characterize the sub-domain (s) of TM1 that are responsible for tumor suppression. In addition, Ras signal transduction pathway are under investigation.

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